Inhibitor discovery by convolution ABPP

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Summary

Activity-based protein profiling (ABPP) has emerged as a powerful proteomic approach to study the active proteins in their native environment by using chemical probes that label active site residues in proteins. Traditionally, ABPP is classified as either comparative or competitive ABPP. In this protocol, we describe a simple method called convolution ABPP, which takes benefit from both the competitive and comparative ABPP. Convolution ABPP allows one to detect if a reduced signal observed during comparative ABPP could be due to the presence of inhibitors. In convolution ABPP, the proteomes are analyzed by comparing labeling intensities in two mixed proteomes that were labeled either before or after mixing. A reduction of labeling in the mix-and-label sample when compared to the label-and-mix sample indicates the presence of an inhibitor excess in one of the proteomes. This method is broadly applicable to detect inhibitors in proteomes against any proteome containing protein activities of interest. As a proof-of-concept, we applied convolution ABPP to analyze secreted proteomes from *Pseudomonas syringae*-infected *Nicotiana benthamiana* leaves to display the presence of a beta-galactosidase inhibitor.

Key words: Activity-based protein profiling (ABPP), convolution ABPP, cyclophellitol-aziridine, galactostatin, *Nicotiana benthamiana*, secreted proteomes, beta-galactosidase, *Pseudomonas syringae*

1. Introduction

Activity-based protein profiling (ABPP) involves chemical probes which label the active site residues in proteins (1). This labeling displays the active state of the targeted proteins. Traditionally, ABPP is classified as either comparative or competitive ABPP (2). Comparative ABPP involves the comparison of the active status of proteins in two or more biological samples (e.g different treatments). In competitive ABPP, a proteome is preincubated with putative inhibitors and subsequently with the activity-based probes to label the non-inhibited proteins. Both of these approaches have emerged as powerful tools to study active proteins in crude proteomes. In this chapter we describe a simple method called convolution ABPP, which takes benefit from both the competitive and comparative ABPP. This method indicates if a reduced signal observed by comparative ABPP is due to the presence of inhibitors.

To illustrate the principle of convolution ABPP, two proteomes (A and B) are shown as example (**Fig. 1**). Labeling of proteomes A and B displays a signal that is reduced in sample B when compared to sample A. Convolution ABPP can be applied in this situation to determine if an excess of inhibitors in proteome B has caused the reduced labeling. Convolution ABPP is a simple, two-step protocol. The first step involves preparation of proteome D, which is a mix of one volume of proteome A with one volume of proteome B. In the second step, proteomes A, B, and D are labeled with an activity-based probe. As a control to display the average signals of samples A and B, one volume of labeled sample A and one volume of labeled sample B are mixed together, resulting in sample C (label-and-mix). In short, the proteomes are mixed-and-labeled (sample D) or labeled-and-mixed (sample C). The fluorescent intensities of labeled proteins in sample C should be the mean of the labeling intensities in samples A and B. Reduced labeling of any protein in sample D when compared to sample C indicates the presence of an inhibitor excess in proteome B. We illustrate this method for detecting inhibitors in two related (biological) samples. However, this approach is broadly applicable for detecting inhibitors in extracts of interest against any proteome whose labeling profile is well-characterized. One important note of caution is that reduced signals can also result from degradation, precipitation or enzymatic deactivation mechanisms other than inhibition. We use protease inhibitor cocktail to prevent degradation but additional experiments e.g. to detect the accumulation and molecular weight of the unlabeled protein is desired to exclude other inactivation mechanisms.

In this chapter, convolution ABPP is illustrated for glycosidases in secreted proteomes (apoplastic fluids) isolated from leaves of the wild tobacco plant *Nicotiana benthamiana*, using JJB70 (**Fig 2a**), a fluorescent activity-based probe for glycosidases (3). JJB70 carries a cyclophellitol-aziridine reactive group and a fluorescent reporter tag. We have shown previously that JJB70 targets 19 different retaining glycosidases in apoplastic fluids of *Nicotiana benthamiana*, belonging to six different glycosyl hydrolase (GH) families (4). Among the different labeled proteins, a 45 kDa signal was identified as a beta-

galactosidase (BGAL) belonging to the GH35 family (4). We have described two experiments to give proof-of-concept of convolution ABPP. In the first experiment we took two identical proteomes (A and B) and added an excess of galactosidase inhibitor to proteome B (**Fig. 2c**). In a second experiment we used convolution ABPP to detect an inhibitor in a biological sample isolated from a pathogen-infected leaf (**Fig. 2d**).

2. Materials

2.1 Infection of *Nicotiana benthamiana* plants with *Pseudomonas syringae*

1. Bacterial stock: *Pto*DC3000 (Δ*hQ*) strain carrying GFP (5) frozen in 7% DMSO

2. Rifampicin (25 mg/mL): dissolve 125 mg of rifampicin in 5ml DMSO, vortex well and store at -20 ºC in 500 µL aliquots.

3. Gentamicin (25 mg/mL): dissolve 125 mg of gentamicin in 5 L sterile water, filter sterilize with 0.22 µM filter unit and store at -20 ºC in 500 µL aliquots.

4. Liquid NYG medium: dissolve 5 g of Bacto™ peptone and 2 g of Bacto™ yeast extract in a 1 L beaker containing 800 mL of milliQ water. Use magnetic stirrer to dissolve. Add 20 mL of glycerol (2%) to the mixture and add milliQ water to a final volume of 1 L. Autoclave the medium at 121 °C and 100 kPa for 15 min.

5. Spectrophotometer and UV cuvettes for measuring optical density (OD) at 600 nM.

6. 1 mL syringe without needle

7. *Nicotiana benthamiana* plants: *N. benthamiana* plants are grown at 22°C and 60% relative humidity under a 12 h light regime. Leaves from 4-week old *N. benthamiana* plants are used for the apoplastic fluid isolation and infection assays.

2.2 Apoplastic fluid isolation

1. Vacuum desiccator.

2. 1 L glass beaker, ice and styropore float.

3. 1 L MilliQ water

4. Apoplastic fluid isolation apparatus (6).

5. Protease inhibitor cocktail: dissolve one Complete Protease Inhibitor Tablet, (Roche, 04693116001) in 2 mL sterile water to give a 25x stock solution.

2.3 Proteomes for ABPP-based mix and label approach

1. 10 mM Galactostatin bisulfite: dissolve 1 mg of galactostatin bisulfite (Santa Cruz Biotechnology, sc-21855) in 411 µL DMSO.

2.4 General procedure for labeling the proteomes

1. 500 mM (10X) MES buffer pH 5.0: dissolve 19.52 g of 2-(*N*-morpholino)ethanesulfonic (MES) in a 200 mL Duran bottle containing 190 mL of milliQ water. Adjust the pH to 5.0 by adding a few drops of 5M NaOH. Add milliQ water to a final volume of 200 mL.

2. 100 µM JJB70: thaw the frozen 10 mM JJB70 stock at room temperature. Dilute this 10 mM stock in DMSO to obtain 100 µM JJB70 can be stored at -20°C until needed.

3. SDS sample buffer (4X): Add 2 g SDS, 10 ml 1 M Tris (pH 6.8) and 10 mL glycerol in a 50 mL falcon tube. Tumble until the reagents are dissolved. Now add 2.5 mL 14.2 M β-mercaptoethanol and add water to a final volume of 25 mL. Finally, add a pinch of Brome Phenol Blue and store in 1 mL aliquots at −20°C.

2.5 Analysis and detection of samples

1. 1M Tris(hydroxymethyl)aminomethane) (Tris) pH 6.8: dissolve 121.14 g of Tris in 1 L Duran bottle containing 800 mL of milliQ water. Adjust the pH to 6.8 with concentrated HCl. Add milliQ water to a final volume of 1 L.

2. 1.5 M Tris(hydroxymethyl)aminomethane) (Tris) pH 8.8: Dissolve 181.7 g of Tris in 1 L Duran bottle containing 800 ml of milliQ water. Adjust the pH to 8.8 with concentrated HCl. Add milliQ water to a final volume of 1 L.

3. 10% Ammoniumpersulfate (APS): dissolve 0.1 g of APS in 1 mL of milliQ water. Prepare the APS solution freshly each time while making protein gels.

4. 10% Sodiumdodecylsulfate (SDS): Dissolve 20 g of SDS in 200 mL of milliQ water.

5. Ready to use 30% (w/v) acrylamide/bis solution (SERVA Electrophoresis GmbH, 10687)

6. TEMED (Sigma-Aldrich, T9281)

7. 12% resolving gel: mix the following components in a conical flask for making eight small protein gels: 18.5 mL water, 22.4 mL 30% (w/v) acrylamide/bis solution, 14 mL 1.5 M Tris pH

8.8, 560 µL 10% SDS, 560 µL 10% APS and 22.4 µL TEMED. Pour the resolving gel solution into the gel cassettes and overlay with 2-Butanol.

8. 6% stacking gel: mix the following components in a conical flask for making at least eight protein gels: 15.7 mL water, 4.8 mL 30% (w/v) acrylamide/bis solution, 3.024 mL 1.0 M Tris pH 6.8, 240 µL 10% SDS, 240 µL 10% APS and 24 µL TEMED. Pour the stacking gel solution onto a polymerized resolving gel and insert the comb with desired well into the cassette.

9. 10X SDS running buffer (10X): dissolve 30 g Tris, 144 g glycine and 10 g SDS in 1 L glass beaker with 800 mL water using a magnetic stirrer. Add water to a final volume to 1 L.

10. Typhoon scanner: Typhoon FLA 9000 scanner (GE Healthcare Life Sciences), or something similar.

2.6 Quantification of fluorescent signals

1. Quantification tool: ImageQuant Version 5.2, Molecular dynamics, or something similar.

Methods

3.1 Infection of *Nicotiana benthamiana* plants with *Pseudomonas syringae* (for Exp.2 only)

1. Revive the *Pto*DC3000(Δ*hQ*) strain carrying GFP (5) from the glycerol stock by plating on LB agar medium containing 25 µg/mL rifampicin and 10 µg/mL gentamicin.

2. Incubate the plate for 48 hours at 28 ºC.

3. Pick a single bacterial colony from the plate and inoculate 10 mL liquid NYG medium containing 25 µg/mL rifampicin and 10 µg/mL gentamicin and grow the bacteria overnight at 28 ºC.

4. Centrifuge the overnight grown bacterial culture at 2000*g* for 15 minutes, remove the supernatant and resuspend the pellet in 4 mL sterile water.

5. Measure the OD at 600 nm and dilute the bacterial suspension with sterile water until $O.D_{600}$ =1 (see note 1)

6. Dilute the bacterial suspension to a concentration of 10^6 bacteria/mL in sterile water.

7. As a control, infiltrate sterilized water into the leaves of 4-week old *Nicotiana benthamiana* plant with a needleless syringe (see note 2) and label the plant as 'A'.

8. Infiltrate the diluted bacteria into leaves of other *N. benthamiana* plants and label the plant as 'B'.

9. At two days post infiltration (2dpi), isolate apoplastic fluids from plants A and B and label the tubes as Proteome A and Proteome B (see section 3.2).

3.2 Apoplastic fluid isolation (Exp.1 and Exp.2)

GMO warning: please be aware that infected leaves contain transgenic bacteria. Take appropriate precautions to sterilize the working area and equipment after the experiment.

1. Take four to six leaves from (infiltrated) *N. benthamiana* plants (see Note 3).

2. Rinse the leaves with water to remove any dirt from the surface of the leaves.

3. Add the leaves, 100 mL ice and 500 mL milliQ water to the 1L beaker and submerge the leaves into the ice cold water with a support. The leaves can be submerged by pushing them down with a piece of styropore that fits tightly in the beaker. In this way the leaves and styropore remain under the water (see Note 4).

4. Place the beaker into a vacuum desiccator and apply about 25 in. Hg vacuum for at least 20 minutes (see Note 5).

5. Stop applying the vacuum and slowly release the vacuum (see Note 6). The water will now enter the leaves.

6. Place the water-infiltrated leaves on a tissue paper and carefully absorb the water droplets from the surface with tissue paper (see Note 7).

7. Roll 6-8 leaves up and carefully place them into the apoplastic isolation device (6) and centrifuge for 25 minutes at 2000*g*, 4 degrees (see Note 8).

8. Collect the apoplastic fluids from the bottom of the apoplastic isolation device, transfer into a 15 mL falcon tube and keep it on ice until further analysis.

9. To an aliquot of the collected apoplastic fluids (e.g. 840 µL) add 25X protease inhibitor cocktail (e.g. 160 µL) to final concentration of 4X (see Note 9).

3.3 Proteomes for convolution ABPP

187 Two experiments will be shown as a proof-of-concept:

	Proteome A (>100 µL)	**Proteome B** (>100 µL)
Exp.1 (**Fig. 2c**)	Apoplastic fluids from untreated plants	Apoplastic fluids from untreated plants + excess galactostatin
Exp.2 (**Fig. 2d**)	Apoplastic fluids from Mock-infiltrated plants	Apoplastic fluids from *P. syringae*-infiltrated plants.

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189 1. Prepare proteome B of Exp.1 by adding galactostatin bisulphite (final concentration 10
190 µM) to the apoplastic fluids of untreated leaves.

191 2. Prepare proteome D by mixing 50 µL of proteome A with 50 µL of proteome B in a 1.5 mL
192 eppendorf tube. Vortex the mixed proteome for 30-60 sec and incubate at room temperature
193 for 30 minutes.

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195 **3.4 General procedure for labeling the proteomes**

196 1. Start the labeling using the following schedule:

	No-probe-control	Sample A	Sample B	Sample D
10X MES buffer (500 mM, pH 5.0)	5 µL	5 µL	5 µL	5 µL
DMSO	1 µL	-	-	-
Probe (100 µM)	-	1 µL	1 µL	1 µL
Protein extracts	44 µL proteome D	44 µL proteome A	44 µL proteome B	44 µL proteome D
Total volume	50 µL	50 µL	50 µL	50 µL

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2. After adding the proteomes, vortex the samples for 5 sec and incubate at room temperature for one hour. Meanwhile cover the samples with aluminium foil or place them in a drawer (see note 10).

3. Stop the labeling reaction by adding 16 µl of 4X SDS sample buffer and heat the samples for 5 minutes at 95ºC in a heat block.

4. Briefly vortex and centrifuge the samples at 16000*g* for 5 seconds.

5. For sample C, mix 5 µL of sample A and 5 µL of sample B in a new 1.5 mL eppendorf tube.

3.5 Analysis and detection of samples A, B, C, D

1. Load 10 µL of the no-probe-control and samples A-D and onto a 12% SDS-PAGE gel.

2. Separate the protein samples at 200V. Meanwhile cover the tank with black plastic or cardboard box (see note 10).

3. Take the gel from the cassette and place the gel in a clean box containing milliQ water.

4. Wash the gel three times with milliQ water for 5 minutes in the dark and immediately proceed to scan the gel with the Typhoon Scanner (See note 11).

5. Detect the fluorescence of labeled proteins by scanning the gel with the Typhoon Scanner using the appropriate settings. For JJB70 we use the 472 nM laser and the BPB1 filter (515-545 nm) and 1000 Volt photo multiplier tube (PMT).

3.6 Quantification of fluorescent signals

1. Save the obtained image in .gel format.

2. To quantify the fluorescence of labeled proteins, open the gel image with ImageQuant quantification tool.

3. Draw a rectangle around the first signal of interest in sample A using the rectangle selection option.

4. Press ‘Ctrl+D' to create new rectangles with the same area as the previous one and place each rectangle on the corresponding band in the B, C and D samples.

5. Select the 'volume' option from the 'analysis' menu and export the results in an excel file.

6. Subtract the values in the volume column with its corresponding background value and divide the obtained value by the area of the selected rectangle. The resulting volume values represent the average fluorescence intensity per unit area in the selected region.

7. Normalize the labeling intensities values to the fluorescence intensity in the sample A and express the values in percentage labeling by multiplying with 100.

8. Perform the steps 4-9 for the other signals of the same gel.

9. Repeat entire assay (steps 3.1-3.5) twice to obtain statistical data.

10. Calculate the standard deviation and standard error of the labeling percentages of three independent experiments and represent the errors bars on the bar graph drawn with the average labeling intensities against the samples.

4. Notes

1. Optical density (OD_{600}) of one represents 10^9 bacteria/mL.

2. Wear protective eye glasses during infiltration. Avoid air bubbles in the syringe during infiltration. Air bubbles in the syringe creates overpressure and causes splashing during infiltration. Air bubbles can also damage the leaf tissues and thereby cause cytoplasmic contaminations.

3. Make sure that the plant is watered well at least 30 minutes before apoplastic fluid isolation. Watering plants opens the stomata which increases access of water into the leaves under vacuum.

4. While submerging the leaves take precaution not to cause any damage to the leaves as this will lead to contamination with cytoplasmic proteins.

5. You will notice that air bubbles emerge after few minutes of applying the vacuum. These bubbles are the air present in the intercellular spaces.

6. Make sure that an entire leaf is completely infiltrated with water. If not, then repeat the vacuum process until the whole leaf is completely infiltrated.

7. Make sure that there are no water droplets left on the surface of the infiltrated leaves. This would dilute the apoplastic proteome.

8. Use slow acceleration and slow de-acceleration settings of the centrifuge to prevent leaf damage caused by centrifugation.

9. If the apoplastic fluids are isolated for protease activity profiling, the addition of protease inhibitor cocktail is not desired.

10. Labeling in the dark reduces photobleaching of the fluorophore.

11. Washing the gel before scanning prevents the formation of Newton-rings while scanning with a Typhoon scanner.

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References:

1. Kolodziejek I, Van der Hoorn RAL (2010) Mining the active proteome in plant science and biotechnology. *Curr Opin Biotechnol* 21, 225-233
2. Cravatt BF, Wright, A. T, and Kozarich, JW (2008) Activity-based protein profiling: from enzyme chemistry to proteomic chemistry. *Annu Rev Biochem* 77, 383-414
3. Kallemeijn, WW, Li KY, Witte MD, Marques ARA, Aten J, Scheij S, Jiang JB, Willems LI, Voorn-Brouwer TM, Van Roomen CPAA, Ottenhoff R, Boot RG, Van den Elst H, Walvoort MTC, Florea BI, Codee JDC, Van der Marel GA, Aerts JMFG, Overkleeft HS (2012) Novel activity-based probes for broad-spectrum profiling of retaining beta-exoglucosidases *in situ* and *in vivo*. *Angew Chem Int Edit* 51, 12529-12533
4. Chandrasekar B, Colby T, Emran Khan Emon A, Jiang J, Hong TN, Villamor JG, Harzen A, Overkleeft HS, Van der Hoorn RAL (2014) Broad-range glycosidase activity profiling. *Mol Cell Proteomics* 13, 2787-2800
5. Misas-Villamil JC, Kolodziejek I, Van der Hoorn RAL (2011) *Pseudomonas syringae* colonizes distant tissues in *Nicotiana benthamiana* through xylem vessels. *Plant J* 67, 774-782
6. Hong TN, Van der Hoorn RAL (2014) DIGE-ABPP by Click Chemistry: pairwise comparison of serine hydrolase activities from the apoplast of infected plants. *Methods Mol Biol* 1127, 183-194

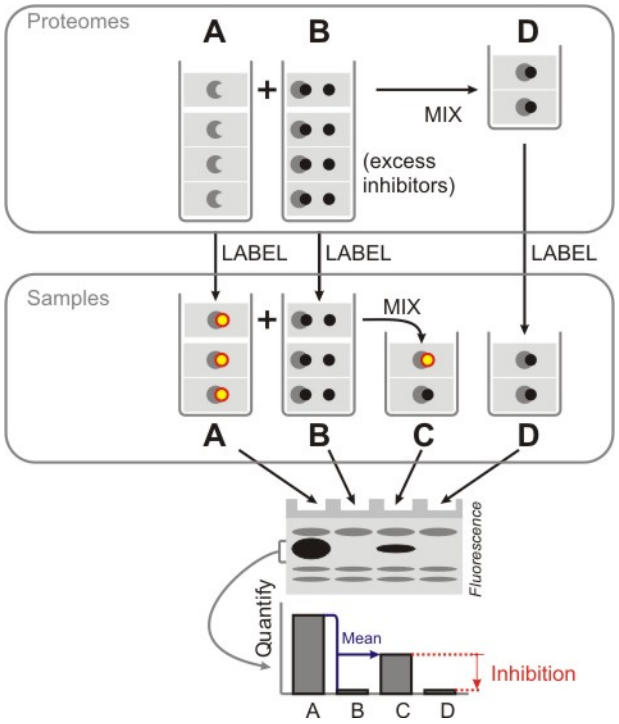

Fig. 1. Procedure of convolution ABPP. This approach can be used if a reduced labeling of a protein is observed in a sample (e.g. any biological treatment, Proteome B) compared to its control (Proteome A). To test if there is an inhibitor excess (black dots) in proteome B, one volume of proteome A and B are mixed together and the mixed proteome (Proteome D) is then labelled (mix-and-label Sample D). As a control for sample D, one volume of labeled samples A and B are mixed together (label-and-mix Sample C). Signals in Sample C should be the average of the signals in Samples A and B. If there is an excess inhibitor in Proteome B then the signal intensity in Sample D will be lower than in Sample C. Black dots, inhibitors; yellow/red dots; activity-based probes; grey moons, enzyme.

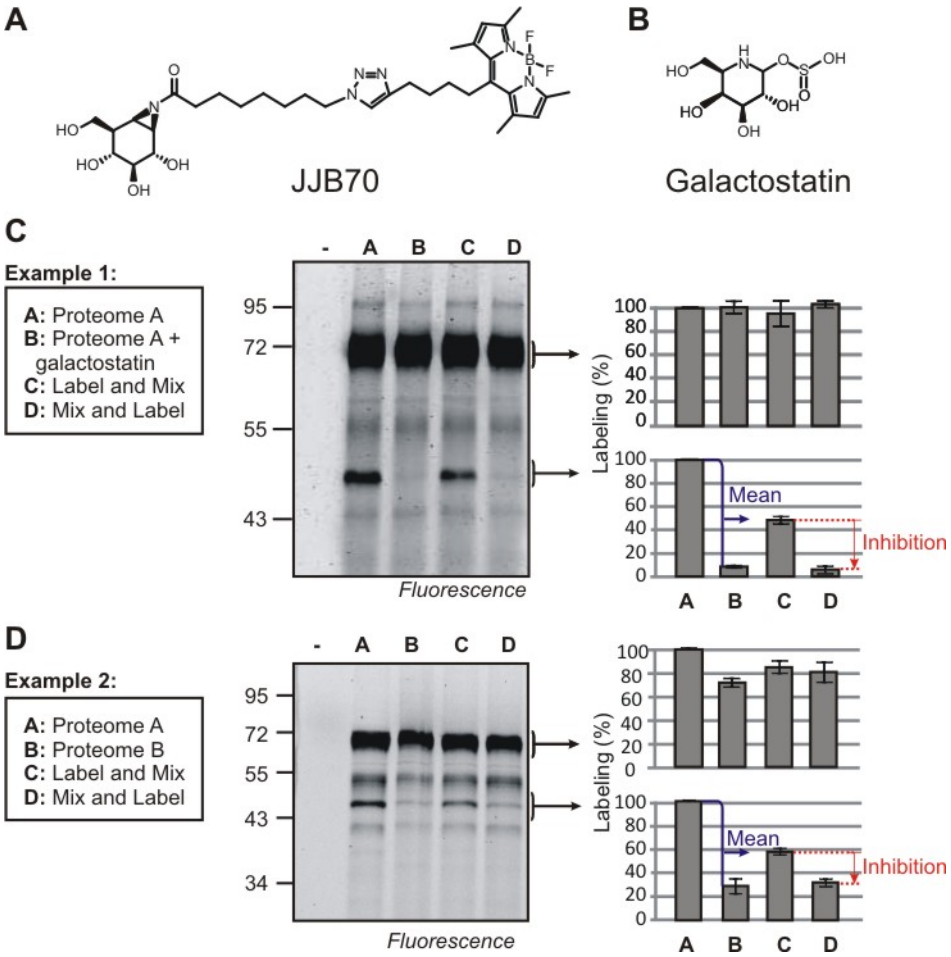

Fig. 2. Proof-of-concept for convolution ABPP. **(a)** Structure of JJB70 probe. JJB70 is a broad range glycosidase probe carrying cyclophellitol-aziridine and bodipy as a reporter tag. **(b)** Structure of galactostatin bisulphite, a beta-galactosidase inhibitor. **(c)** Convolution ABPP with a proteome containing an excess of a known inhibitor. Proteome A is an apoplastic fluid from untreated *N. benthamiana* leaves and proteome B is the same proteome containing 10 µM galactostatin bisulphite. Equal volumes of proteome A and proteome B were mixed together, resulting in proteome D. Proteomes A, B and D were labeled with 2 µM JJB70 for one hour. After labeling, one volume of labeled samples A and B were mixed together (label-and-mix Sample C). The labeled samples A, B, C, D were separated on a protein gel, detected by in-gel fluorescent scanning and the labeling intensity was quantified. Error bars indicate the standard error of means from three independent experiments. **(d)** Convolution ABPP indicates the presence of inhibitors in *P. syringae*-infected *N. benthamiana* leaves. Proteome A represents apoplastic fluids from the mock-infiltrated *N. benthamiana* leaves and Proteome B represents apoplastic fluids from *P. syringae*-infiltrated *N. benthamiana* leaves. The labeled samples A, B, C (label-and-mix) and D (mix-and-label) were prepared and analyzed as in (c). Error bars indicate the standard error of means from three independent experiments.